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NOTES

Selection of H5N1 Influenza Virus PB2 during Replication in Humans▽

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Highly pathogenic H5N1 influenza viruses continue to cause concern, even though currently circulating strains are not efficiently transmitted among humans. For efficient transmission, amino acid changes in viral proteins may be required. Here, we examined the amino acids at positions 627 and 701 of the PB2 protein. A direct analysis of the viral RNAs of H5N1 viruses in patients revealed that these amino acids contribute to efficient virus propagation in the human upper respiratory tract. Viruses grown in culture or eggs did not always reflect those in patients. These results emphasize the importance of the direct analysis of original specimens.

Given the continued circulation of highly pathogenic H5N1 avian influenza viruses and their sporadic transmission to humans, the threat of a pandemic persists. However, for H5N1 influenza viruses to be efficiently transmitted among humans, amino acid substitutions in the avian viral proteins may be necessary.

Two positions in the PB2 protein affect the growth of influenza viruses in mammalian cells (3, 11, 18): the amino acid at position 627 (PB2-627), which in most human influenza viruses is lysine (PB2-627Lys) and most avian viruses is glutamic acid (PB2-627Glu), and the amino acid at position 701. PB2-627Lys is associated with the efficient replication (16) and high virulence (5) of H5N1 viruses in mice. Moreover, an H7N7 avian virus isolated from a fatal human case of pneumonia possessed PB2-627Lys, whereas isolates from a nonfatal human case of conjunctivitis and from chickens during the same outbreak possessed PB2-627Glu (2).

The amino acid at position 701 in PB2 is important for the high pathogenicity of H5N1 viruses in mice (11). Most avian influenza viruses possess aspartic acid at this position (PB2-701Asp); however, A/duck/Guangxi/35/2001 (H5N1), which is highly virulent in mice (11), possesses asparagine at this position (PB2-701Asn). PB2-701Asn is also found in equine (4) and swine (15) viruses, as well as some H5N1 human isolates (7, 9). Thus, both amino acids appear to be markers for the adaptation of H5N1 viruses in humans (1, 3, 17).

TABLE 1. H5N1 virus specimens analyzed

Specimen ID[a]	Collection site	Date of collection	Patient age (yr)	Patient gender	Patient outcome	Virus strain previously reported and reference[b]
HN3040I	Pharynx	6 January 2004	10	Male	Fatal	A/Vietnam/1203/04 (12)
HN3040II	Trachea	7 January 2004	10	Male	Fatal	A/Vietnam/1204/04 (12)
HN3030I	Trachea	29 December 2003	5	Male	Fatal	A/Vietnam/3030/04 (19)
HN3030II	Trachea	31 December 2003	5	Male	Fatal	
HN3047III	Pharynx	21 January 2004	23	Female	Fatal	A/Vietnam/3047III/04 (19)
HN3062	Pharynx	16 January 2004	19	Male	Fatal	

[a] HN3040I and HN3040II were collected from the same patient, and HN3030I and HN3030II were collected from the same patient. ID, identification.
[b] These viruses were previously isolated by another group (12) and us (19).

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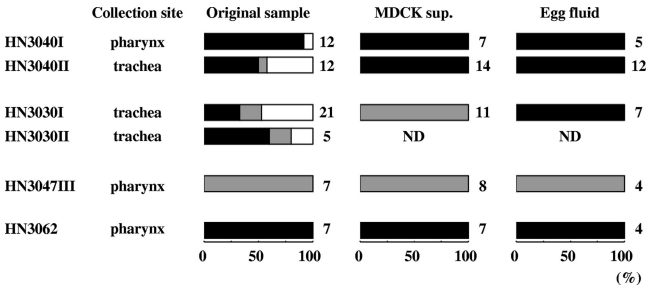

FIG. 1. Virus populations in the original specimens, the supernatants of MDCK cells, and the allantoic fluid of embryonated chicken eggs characterized by their PB2 genes. The PB2 genes from each sample were cloned into plasmids by reverse transcriptase PCR, ligation, and transformation. The plasmids were purified from independent *E. coli* transformants and their nucleotide sequences analyzed. The viruses possessing PB2-627Lys and PB2-701Asp (human type) are shown as black bars, those possessing PB2-627Glu and PB2-701Asn (also human type) as gray bars, and those possessing PB2-627Glu and PB2-701Asp (avian type) as white bars. The number of clones analyzed for each sample is indicated to the right of the bars. Results for the original specimens, the supernatants of MDCK cells (cultured in single wells), and the allantoic fluid of embryonated chicken eggs (three eggs for HN3040I, -3040II, and -3047 and one egg for HN3030I and -3062 were used; allantoic fluid from the same samples were pooled) are shown in the left, middle, and right columns, respectively. The MDCK cells were infected with 1,000-fold diluted HN3040I, 10-fold diluted HN3040II, 10-fold diluted HN3030I, original (nondiluted) HN3030II, 10-fold diluted HN3047III, and 1,000,000-fold diluted HN3062. The eggs were infected with 1,000-fold diluted HN3040I, 100-fold diluted HN3040II, 10,000-fold diluted HN3028I, 1,000-fold diluted HN3028II, 100-fold diluted HN3047III, and 10,000,000-fold diluted HN3062. ND, virus not detected.

Massin et al. (13) reported that the amino acid at PB2-627 affects viral RNA replication in cultured cells at low temperatures. Recently, we demonstrated that viruses, including those of the H5N1 subtype, with PB2-627Lys (human type) grow better at low temperatures in cultured cells than those with PB2-627Glu (avian type) (6). This association between the PB2 amino acid and temperature-dependent growth correlates with the body temperatures of hosts; the human upper respiratory tract is at a lower temperature (around 33°C) than the lower respiratory tract (around 37°C) and the avian intestine, where avian influenza viruses usually replicate (around 41°C). The ability to replicate at low temperatures may be crucial for viral spread among humans via sneezing and coughing by being able to grow in the upper respiratory organs. Therefore, the Glu-to-Lys mutation in PB2-627 is an important step for H5N1 viruses to develop pandemic potential.

However, there is no direct evidence that the substitutions of PB2-627Glu with PB2-627Lys and PB2-701Asp with PB2-701Asn occur during the replication of H5N1 avian influenza viruses in human respiratory organs. Therefore, here, we directly analyzed the nucleotide sequences of viral genes from several original specimens collected from patients infected with H5N1 viruses.

Mixed PB2 population in original specimens. To investigate the significance of PB2-627Lys and PB2-701Asn in H5N1 viruses for replication in humans, we extracted viral RNAs from six original specimens (once per specimen) using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNAs were reverse transcribed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and an oligonucleotide complementary to the 12-nucleotide sequence at the 3′ end of the viral RNA and amplified by PCR with Pwo DNA polymerase (Roche, Basel, Switzerland) and primers specific for the PB2 segment of the H5N1 influenza virus (sequences available upon request). The PCR products were gel purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned into pCR-Blunt II-TOPO (Invitrogen) by TOPO cloning technology (Invitrogen) and transformed into TOP10 cells (Invitrogen). *Escherichia coli* clones were picked, and the nucleotide sequences of the viral genes that cover the regions that include PB2-627 and -701 were analyzed. All experiments with infectious H5N1 viruses were performed under biosafety level 3 containment.

We first examined two different specimens obtained from the same patient (HN3040), believed to be directly infected with H5N1 avian viruses. One was a pharyngeal swab collected from the upper respiratory tract; the other was a tracheal aspirate from the lower respiratory tract (Table 1). Maines et al.

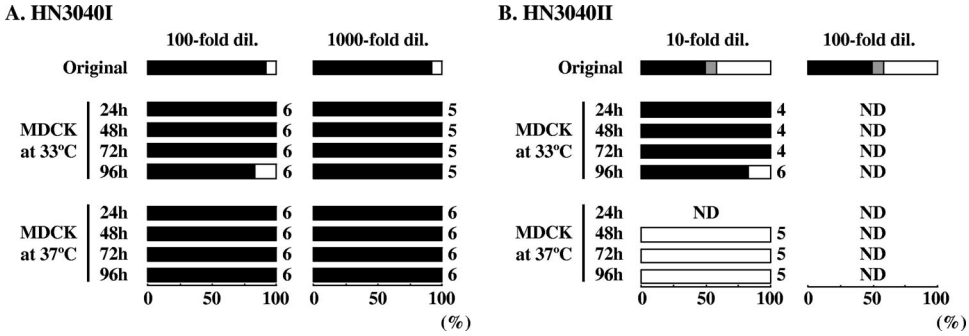

FIG. 2. Alteration of virus populations characterized by the PB2 gene following their propagation in MDCK cells. The original specimens HN3040I (A) and HN3040II (B) diluted with minimal essential medium containing 0.3% bovine serum albumin at the indicated dilutions were inoculated into MDCK cells. The culture supernatants, incubated at 33°C (top rows) or 37°C (bottom rows), were independently harvested at the indicated times after inoculation and subjected to sequence analysis of the viral PB2 gene. The ratio of the viruses that had PB2-627Lys and PB2-701Asp, PB2-627Glu and PB2-701Asn, and PB2-627Glu and PB2-701Asp are represented by black (human type), gray (human type), and white (avian type) bars, respectively. The number of clones analyzed for each sample is indicated to the right of the bars. The results for the supernatants of the MDCK cells (cultured in single wells) infected with the 100- and 1,000-fold diluted HN3040I (A) and the 10- and 100-fold diluted HN3040II (B) from MDCK cells are shown. ND, virus not detected.

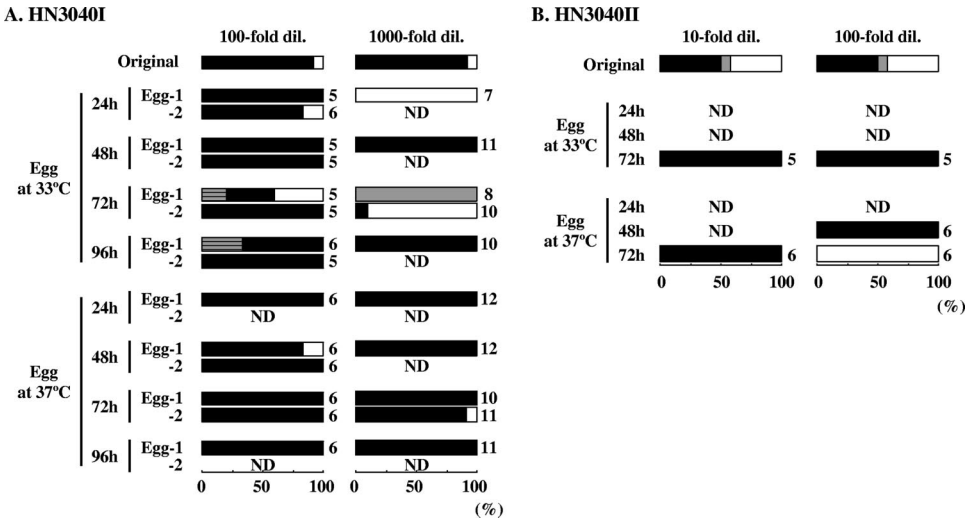

FIG. 3. Alteration of virus populations characterized by the PB2 gene following their propagation in embryonated chicken eggs. The original specimens HN3040I (A) and HN3040II (B) diluted with phosphate-buffered saline as indicated were inoculated into embryonated chicken eggs. The allantoic fluid of the eggs, incubated at 33°C (upper rows) or 37°C (lower rows), was independently harvested at the indicated times after inoculation and subjected to sequence analysis of the viral PB2 gene. The ratio of the viruses that had PB2-627Lys and PB2-701Asn, PB2-627Lys and PB2-701Asp, PB2-627Glu and PB2-701Asn, and PB2-627Glu and PB2-701Asp are represented by the striped (human type), black (human type), gray (human type), and white (avian type) bars, respectively. The number of clones analyzed for each sample is indicated to the right of the bars. The results for the 100- and 1,000-fold diluted HN3040I (A) and the 10- and 100-fold diluted HN3040II (B) are shown. ND, virus not detected.

(12) previously isolated H5N1 viruses with PB2-627Lys (A/Vietnam/1203/04 [H5N1]) from sample HN3040II and PB2-627Glu (A/Vietnam/1204/04 [H5N1]) from HN3040I (both possess PB2-701Asp) (Table 1). A direct analysis of the viral RNAs in these two original specimens revealed avian-type (no PB2-627Lys or PB2-701Asn) and human-type (PB2-627Lys or PB2-701Asn) viruses in each specimen (Fig. 1). Mixed PB2 populations in two other specimens from patient HN3030 were also observed (Fig. 1). These results suggested that although it is possible that the viruses possessing the human-type PB2 genes were already circulating in birds, human-adapted viruses likely emerged during replication in humans.

Most of the virus in HN3040I possessed human-type PB2 (11/12 clones), compared to 7/12 clones in HN3040II (Fig. 1). In the other two specimens collected from the upper respiratory tract, HN3047III and HN3062, we found only human-type PB2 amino acids (Fig. 1). On the other hand, 47% and 20% of the specimens from the lower respiratory tract, HN3030I and HN3030II, respectively, contained avian-type PB2 (Fig. 1). These results demonstrate that the human-type PB2-627Lys and PB2-701Asn are selected during virus replication in the upper respiratory tract of humans.

Virus selection during propagation in MDCK cells and embryonated chicken eggs. Generally, MDCK cells are used for virus isolation from mammals (except horses [8]), and embryonated chicken eggs are used for virus isolation from birds. Human viruses with hemagglutinin mutations are selected during propagation in eggs (10, 14). Thus, these procedures may misrepresent virus populations in humans. To test this possibility, we inoculated specimens (Table 1) into MDCK cells and chicken eggs, incubated them at 37°C for 2 to 5 days, and compared the PB2 gene in the propagated viruses with the original specimens (Fig. 1). We found no viruses with avian-type PB2 in any of the specimens, indicating that under these conditions, H5N1 viruses with avian-type PB2 do not propagate in MDCK cells or chicken eggs.

To reveal the factor(s) that caused this discordance between the original specimens and the viral stocks, we inoculated HN3040I and HN3040II into MDCK cells (Fig. 2) and chicken eggs (Fig. 3), incubated them at 33°C and 37°C, harvested the cultured supernatants and allantoic fluids at various time points, and analyzed the PB2 gene in the propagated viruses.

Although most of the viruses grown in MDCK cells possessed human-type PB2, all of the viruses grown in these cells with HN3040II at a 10-fold dilution at 37°C, but not at 33°C, possessed the avian-type PB2 (Fig. 2). These results show that avian-type viruses are selected at higher temperatures in MDCK cells. The inconsistent results with HN3040II-inoculated MDCK cells at 37°C (Fig. 1 and 2B) may reflect low virus titer in the original specimen. A limited number of virions containing only human-type PB2 may have been inoculated into the cells. This also explains the lack of virus with the 100-fold-diluted HN3040II (Fig. 2B, right column).

Viruses grown in eggs were more heterogeneous than those grown in MDCK cells with HN3040 samples (Fig. 2 and 3). Since the viruses with PB2-701Asn were not detected in the original specimen, the detection of such viruses at 33°C but not at 37°C in eggs suggests that PB2-701 may promote their growth under this condition.

Our direct analysis of the viral RNAs from the original patient specimens showed that PB2-627Lys and PB2-701Asn of the H5N1 avian influenza viruses were independently selected during replication in the human respiratory tract. The direct sequencing of the samples identified a mixed population with avian-type and human-type PB2, suggesting that viruses with human-type amino acids arose under selective pressures

in humans. We also demonstrated that a PB2 gene population grown in either cell culture or eggs does not always reflect that in patients. A direct analysis of the original specimens is required for accurate information on the viral genome and to monitor for prepandemic viruses.

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